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DNA Methylation Patterns in Human Tissues of Uniparental Origin Using a Zinc-Finger Gene (*ZNF127*) From the Angelman/Prader-Willi Region

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In order to further our understanding of the epigenetic modifications of DNA and its role in imprinting, we examined DNA methylation patterns of human tissues of uniparental origin. We used complete hydatidiform moles (CHM), which are totally androgenetic conceptions, to examine the paternal methylation pattern in the absence of a maternal contribution and we used ovarian teratomas to represent the maternal counterpart. We carried out an analysis of DNA methylation of a gene which has been shown to contain sites which are differentially methylated in a parent-specific fashion. The gene, *ZNF127*, is located on chromosome 15q11-q13 in the region associated with Prader-Willi and Angelman syndromes. The parent-of-origin DNA methylation has been postulated to reflect the presence of an imprint and recent studies have confirmed that *ZNF127* is differentially expressed only from the paternal chromosome. We identified a unique pattern of hyper- and hypomethylated sites in androgenetic conceptions which was nearly identical to the paternal pattern found in sperm. This may represent the paternal germ-line methylation imprint. We also studied partial hydatidiform moles, non-molar triploid conceptions, normal chorionic villi, and somatic tissue. These all demonstrated a modified DNA methylation pattern characteristic of normal chorionic villi with only limited findings of the imprint. Our results suggest that human androgenetic conceptions may provide an excellent model to analyze epigenetic DNA modifications, such as methylation, in imprinted genes. The paternal allele-specific methylation imprint will also be useful clinically to confirm the androgenetic nature of suspected molar conceptions in which parental blood samples may not be available.

KEY WORDS: DNA methylation, genomic imprinting, complete hydatidiform mole

INTRODUCTION

Classical Mendelian genetics assumes that the parental origin of a gene will not influence its expression. However, this does not appear to be the case with a small number of genes which may be involved in embryonic and postnatal development. This phenomenon, known as genomic imprinting, is thought to play a role in development by allowing certain parental genes to be "marked" and then differentially expressed at different stages of development. The mechanism of genomic imprinting must modify the DNA in a way that requires regulatory factors, expressed in the cell at later times, to recognize the imprint and selectively activate or inactivate one allele.

The functional behavior of transgenes in mice first suggested that DNA methylation may be involved in imprinting. Several studies have demonstrated that some transgenes are reversibly methylated following passage through the male or female germline. The pattern of methylation is consistent with the parental origin of the transgene in each subsequent generation [Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987; Chaillet et al., 1991]. The development of uni-

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parental disomies for specific chromosomal regions in the mouse has shown that there are distinct regions of the genome which are required from each parent for normal embryonic development [Cattanach and Kirk, 1985; Cattanach, 1986]. These critical regions on the mouse chromosomes have provided a framework for identifying individual genes which are imprinted.

Stöger et al. [1993] recently presented compelling evidence that DNA methylation is directly involved in genomic imprinting. Examination of methylation patterns of the mouse insulin-like growth factor-2 receptor (*Igf2r*) gene indicated that two regions were differentially methylated in a parent-specific manner. The first region, which contained the transcription start site, was methylated on the silent paternal allele. This methylation was acquired after fertilization and probably occurred secondary to inactivation. In region 2, the active maternal allele demonstrated methylation of specific CpG sites which were unmethylated on the inactive paternal allele. This region is located 27 Kb downstream of the promoter and may be the signal which allows this gene to be expressed. This methylation pattern was also seen in the maternal gamete, suggesting that the methylation imprint was established during gametogenesis. The absence of this methylation pattern on the paternal allele may play a role in the inactivation of that allele. This pattern was also seen at the morula and blastocyst stages of embryonic development and in embryonic stem cells [Stöger et al., 1993].

The phenomenon of genomic imprinting has also been observed in humans. The classical examples are Prader-Willi and Angelman syndromes. These two syndromes most commonly arise due to a deletion of chromosome 15 region q11-q13 [Ledbetter et al., 1981]. Cytogenetically the deletions are identical; however, in Prader-Willi syndrome (PWS) it is the paternal chromosome that is deleted [Butler et al., 1986] and in Angelman syndrome (AS) it is the maternal chromosome that is deleted [Kaplan et al., 1987; Knoll et al., 1989]. The occurrence of uniparental disomy for chromosome 15 also contributes to the cause of both of these syndromes [Nicholls et al., 1989; Malcolm et al., 1991]. There have been reports of human uniparental disomy playing a role in the cause of several other genetic disorders [Spence et al., 1988]. The effects of imprinting have also been observed in certain types of tumors and in some cases imprinting has been disrupted [Rainer and Feinberg, 1994].

The study of the unmodified germ-line imprint in humans has been hindered by the inability of investigators to examine the maternal and paternal alleles individually during development. The *ZNF127* and *SnrpN* genes were amenable to the study of imprinting because the methylation patterns of the individual parental alleles could be determined by examining the peripheral blood leukocytes (PBLs) from patients with PWS and AS [Driscoll et al., 1992; Glenn et al., 1993b; Jong et al., in preparation]. This is a unique situation in the human genome, in which two distinct clinical abnormalities result from an absence of the same chromosomal region. Investigators currently postulate that the 2 syndromes result from deletions of at least two different imprinted genes which are closely linked and located within the critical region. However, this system is limited to examining only genes on chromosome 15. An adequate model for examining the human genome for other imprinted genes has not been found. The use of uniparental human conceptions and germ cell tumors may provide us with the material necessary for locating and analyzing imprinted genes and their effects in humans.

Complete hydatidiform moles (CHM) are naturally occurring androgenones [Kajii and Ohama, 1977]. They most often arise when an anucleate egg is fertilized by a single haploid sperm which undergoes endoreduplication to give rise to a diploid, homozygous, androgenetic conception. The grossly abnormal phenotype of complete hydatidiform moles is most likely the result of abnormal expression of one or more imprinted genes acting early in embryonic development. They are characterized by universal swelling of the chorionic villi, trophoblastic hyperplasia, and the absence of a fetus. Alternately, ovarian teratomas appear to represent parthogenones that arise from activated female germ cells and undergo partial embryogenesis without a paternal contribution [Linder et al., 1975; Surti et al., 1990; Deka et al., 1990]. A teratoma can give rise to tissues derived from all three germ cell layers and often yield fully differentiated structures, such as teeth, bone, skin, and hair.

Here we report on the use of complete hydatidiform moles and ovarian teratomas to examine the DNA methylation patterns of *ZNF127* to determine whether they maintain the parental allele-specific patterns as seen in PBLs from AS/PWS patients [Driscoll et al., 1992].

MATERIALS AND METHODS

Specimen Collection

This study used tissue samples collected as part of a larger study of the genetics of trophoblast disease and gynecological tumors at the Department of Pathology, Magee Womens Hospital, Pittsburgh, Pennsylvania. Each sample of chorionic villous was carefully cleaned under a dissecting microscope to remove blood clots and decidua. The morphology was noted and a piece was submitted for histology. Complete and partial hydatidiform moles were identified by criteria as described by Szulman and Surti [1978a,b]. Primary cultures were set-up on each case for cytogenetic analysis, DNA was generally extracted from freshly dissociated tissue, and the remaining tissue was frozen for future analysis.

Molecular Studies

Genomic DNA was isolated using both a nonorganic procedure [Miller et al., 1988] and by standard phenol/chloroform extraction [Blin and Stafford, 1976]. The parental origin of hydatidiform moles was determined by chromosomal heteromorphisms, as well as restriction fragment length polymorphism analysis of 5 loci (YNZ2, *D1S57;* YNH24, *D2S44;* 68RS2.0, *RB1;* EKMDA2.1, *D16S83;* YNZ22, *D17S5*) [Nakamura et al., 1987a,b, 1988a,b; Wolff et al., 1988] and the 3′ hypervariable region of the apolipoprotein B gene was ampli-

fied by the polymerase chain reaction (PCR) using the oligonucleotide primers and conditions described by Boerwinkle et al. [1989]. The technique for nitrocellulose blotting was a modified Southern method [Desai et al., 1978]. Our hybridization protocol was modified from previously described techniques [Kunnath and Locker, 1982]. The DNA methylation status of this region was assessed by digesting the DNA with methylation-sensitive restriction enzymes HpaII and HhaI, as previously described [Driscoll et al., 1992]. The blots were hybridized with the cDNA DN34 (*D15S9*) which was cloned from a human fetal brain library [Neve et al., 1986; Tantravahi et al., 1989].

RESULTS

The objective was to examine the DNA methylation pattern of complete hydatidiform moles and ovarian teratomas and compare them to the patterns seen in normal chorionic villi. We wanted to determine whether androgenetic complete moles and parthenogenetic ovarian teratomas displayed the paternal and maternal allele-specific methylation patterns, respectively. A total of 16 CHM samples were analyzed. Ten were confirmed to be completely androgenetic in origin using both chromosome and DNA analysis of the CHM and parental blood samples (Surti, unpublished data). The origin of the remaining 6 samples could not be determined because parental blood was not available, but histopathological examination confirmed the diagnosis. The 3 benign ovarian teratomas were karyotyped and characterized according to their mechanism of origin [Surti et al., 1990].

Analysis of the degree of DNA methylation of *ZNF127* was carried out using Southern blot analysis with the methylation sensitive restriction enzymes HhaI and HpaII using the cDNA probe DN34, as previously described for peripheral blood leukocytes [Driscoll et al., 1992]. We analyzed a total of 49 samples, which included 16 complete hydatidiform moles (CHM), 5 partial hydatidiform moles (PHM), 4 non-molar triploids (NMT), 9 normal chorionic villous samples, 11 other samples (sperm, 1; adult ovary, 3; benign ovarian teratomas, 3; malignant immature testicular teratoma, 1; choriocarcinoma cell lines, 3), and 4 adult peripheral blood leukocytes samples. We included the partial moles and non-molar triploids because they contain additional paternal and maternal haploid sets of chromosomes, respectively. The sperm and teratomas (ovarian and testicular) were analyzed because of their germ cell origin.

Restriction enzyme analysis of normal control DNA from adult peripheral blood leukocytes with EcoRI + HpaII yielded 4 major bands: 5.2 Kb (E2-E1), 4.3 (M5-E1), 4.0 (M4-E1), and 3.5 (M1-E1) (Fig. 1). The 4 bands resulted from partial digestion of HpaII sites M1, M4, and M5 and no digestion at sites M2 and M3[a–e], which were completely methylated. The multiple M3 sites are too close together to be analyzed individually [Jong et al., in preparation]. In normal PBLs, most of cells were totally methylated at the HpaII sites, as demonstrated by the intense 5.2 Kb band (Fig. 1, lane 7). The normal chorionic villous samples demonstrated a reduction in the overall level of methylation as indicated by the reduced intensity of the 5.2 and 4.3 Kb bands and an increase in the 3.5 Kb band (Fig. 1, lane 4). The CHM demonstrated a DNA methylation pattern that was not seen in any other chorionic villous samples (Fig. 1, lanes 1–3). Thirteen of 15 CHM demonstrated a pattern with only 2 bands, a prominent band at 3.5 Kb (E1-M1) and a second band at 0.9 Kb (E2-M4) (Fig. 1). The other bands were almost completely absent, indicating that the CHMs were almost totally unmethylated at the internal HpaII sites. The M5 site must be totally methylated in order to give rise to the 0.9 Kb band. The smaller bands resulting from digestion of these sites could not be resolved. The 14th and 15th CHMs had a different pattern, showing a less prominent 3.5 Kb band and an additional band at 4.0 Kb of equal intensity. The appearance of this second band indicated that the M1 site was methylated in some of the cells (data not shown). This variation in the methylation most likely reflects maternal cell contamination of the DNA sample, or may suggest that these two conceptions were not CHM, although this is less likely based on the histologic diagnosis. The parental origin of these two specimens could not be confirmed because parental DNA was not available.

The DNA isolated from the ovarian teratomas and corresponding normal adult ovary demonstrated a DNA methylation pattern which was similar to peripheral blood leukocytes (Fig. 1, lane 6). The nature of the differentiated tissues observed in the ovarian tera-

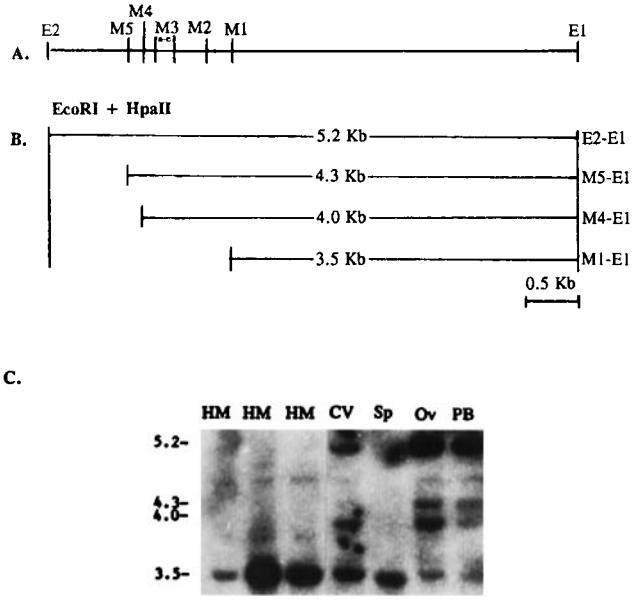

Fig. 1. Restriction map and DNA methylation pattern of HpaII sites in the *ZNF127* locus. **A:** Restriction map of *ZNF127* with the location of five HpaII sites within a 5.2 Kb EcoRI fragment. **B:** Sizes of the four bands produced when the DNA is digested with EcoRI + HpaII, when each individual HpaII site is unmethylated. Sites M3[a–e] and M4 cannot be differentiated due to their close proximity to each other. **C:** Methylation pattern of DNA digested with EcoRI + HpaII and probed with ^{32}P-DN34 cDNA. HM = complete hydatidiform mole; CV = normal (46,XX) age-matched chorionic villi; Sp = sperm; Ov = adult ovarian tissue; PB = peripheral blood lymphocytes from a normal adult male; E = EcoRI; M = MspI/HpaII.

tomas suggests that the germ-line pattern has probably been obscured by the developmental methylation changes associated with differentiation. The DNA from the adult ovarian tissue was extracted from the outer cell wall surrounding the teratoma and did not contain any germ cells. Additional studies using female germ cells from fetal ovaries may yield more information about the maternal allele-specific methylation pattern at this locus. The testicular teratoma, on the other hand, demonstrated a paternal pattern similar to that seen in CHM. These results suggest that this tumor was derived from a male germ cell and that the subsequent cells maintained the paternal allele-specific methylation pattern at this locus.

We also examined the DNA methylation pattern of sperm, in order to determine whether the pattern was similar to that observed in CHM. The sperm had the same DNA methylation pattern as the CHM. This is especially interesting since sperm DNA has been shown to be hypermethylated at most loci examined previously [Kunnath and Locker, 1982; Monk et al., 1987; Trasler et al., 1990; Driscoll and Migeon, 1990]. These results suggested that the paternally derived allele may be almost totally unmethylated in the region assayed by HpaII.

In order to address this question we analyzed the DNA methylation status of 5 HhaI sites within the same region of DNA. These sites were analyzed by double digestion with HindIII and HhaI [Driscoll et al., 1992]. Normal control DNA from adult peripheral blood leukocytes yielded 6 bands: 4.5 Kb (H2-H1), 3.5 (Hh4-H1), 3.1 (H2-Hh1), 2.9 (Hh3-H1), 2.6 (Hh2-H1), 2.0 (H2-Hh2)[1] (Fig. 2). These bands are the result of cell to cell variations in the methylation pattern. The CHM demonstrated 4 bands at 3.5, 2.6, 1.0, and 0.6 Kb (Fig. 2C, lanes 1–3) in 14 of 16 cases (1.0 and 0.6 Kb bands not shown). The more prominent 2.6 Kb band corresponded to Hh2-H1 and indicated that Hh2 was almost totally unmethylated, while Hh1 remained totally methylated, in the majority of cells. The weaker 3.5 Kb band corresponded to Hh4-H1 and indicated that a smaller number of cells were methylated at sites Hh1, Hh2, and Hh3, but not Hh4. The other lower molecular weight bands, 1.0 and 0.6 Kb, may correspond to fragments H2-Hh4, Hh4-Hh3, and H2-Hh5, Hh5-Hh4, respectively. The presence of the 2.6 and 3.5 Kb bands was a significant finding, because it indicated that the segment was not completely unmethylated. Therefore, despite the low level of methylation seen at the HpaII sites in CHM, some sites remain consistently methylated. Most significantly this pattern was similar to that seen in sperm DNA. These results clearly support the hypothesis that the paternal allele-specific methylation pattern is maintained in CHM. The ovarian teratoma demonstrated the same methylation pattern as biparental normal PBLs, indicating that the exclusive uniparental maternal pattern is not maintained. The reason for this observation is not clear, except that this pattern may be obscured in the differentiated tissues

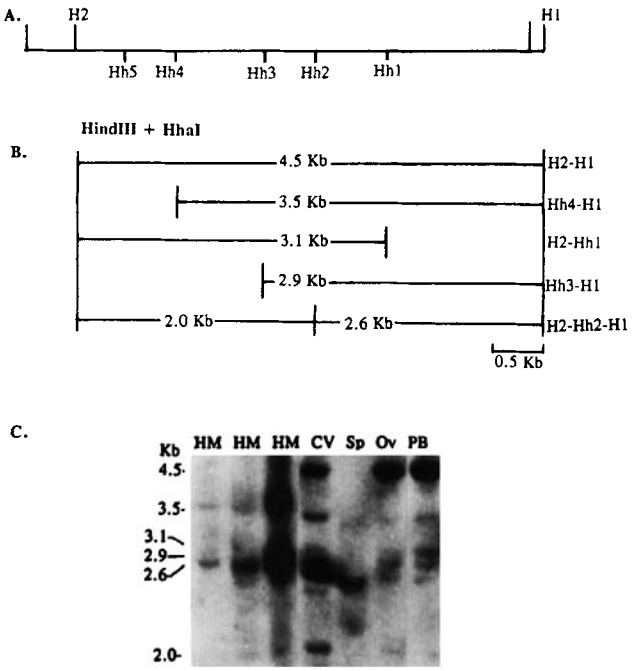

Fig. 2. Restriction map and DNA methylation pattern of HhaI sites in the *ZNF127* locus. **A:** Restriction map of *ZNF127* with the location of five HhaI sites within a 4.5 Kb HindIII fragment. **B:** Sizes of the six bands produced when the DNA is digested with HindIII + HhaI, when each individual HhaI site is unmethylated. **C:** Methylation pattern of DNA digested with HindIII + HhaI and probed with ^{32}P-DN34 cDNA. HM = complete hydatidiform mole; CV = normal (46,XX) age-matched chorionic villi; Sp = sperm; Ov = adult ovarian tissue; PB = peripheral blood lymphocytes from a normal adult male: H = HindIII; Hh = HhaI.

present in teratomas. More cases need to be analyzed to see if ovarian teratomas resulting from different meiotic origins (Type I or Type II) will show the same pattern [Surti et al., 1990].

In addition to the androgenetic CHM and ovarian teratomas, we examined the DNA methylation patterns of other abnormal conceptions with unequal parental contributions. We analyzed 5 partial hydatidiform moles and 4 non-molar triploids (data not shown). The presence of an extra parental contribution was reflected in the methylation patterns of the PHM's. They displayed all of the characteristic bands seen in normal chorionic villi, except that the 3.5 Kb HpaII band and 2.6/3.5 Kb HhaI bands were more prominent. This was not seen in non-molar triploids which appeared to have a methylation pattern which was more similar to adult PBLs. The PHM and NMT patterns probably retain their germline methylation patterns; however, they have been somewhat obscured by developmental methylation changes which occur during normal embryogenesis. Figure 3 illustrates the methylation patterns of the HhaI and HpaII sites which we examined within the *ZNF127* gene sequence. There is clearly a paternal-specific methylation pattern which is consistently seen in CHM and sperm that is similar but not identical to the pattern first described in the PBLs of AS patients [Driscoll et al., 1992].

[1]Sequence data suggests that these bands may have multiple origins [Jong et al., in preparation].

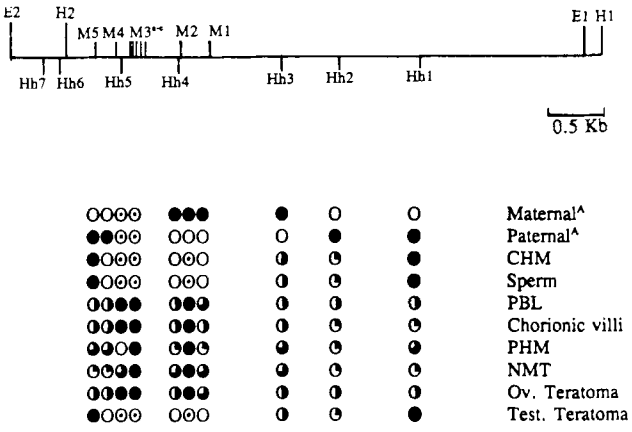

Fig. 3. Methylation status of HpaII and HhaI sites mapped to the *ZNF127* locus. Each line represents a composite of the pattern observed in a single type of specimen. Each circle represents a distinct methylation site. The shading of the circles indicates the average level of methylation seen at each site. E = EcoRI; H = HindIII; M = MspI/HpaII; Hh = HhaI; ○ = totally unmethylated; ● = totally methylated; ◔,◑,◕ = partially methylated; ⊙ = methylation cannot be determined; PBL = peripheral blood leukocytes; CHM = complete hydatidiform mole; PHM = partial hydatidiform mole; NMT = non-molar triploid; Ov = ovarian; Test = testicular; Maternal^A = methylation pattern seen in PBLs from Prader-Willi syndrome patients with either a paternal deletion of 15q11q13 or maternal uniparental disomy; Paternal^A = methylation pattern seen in PBLs from Angelman syndrome patients with either a maternal deletion or paternal uniparental disomy. A = determined by Driscoll et al. [1992].

DISCUSSION

The analysis of genomic imprinting must be divided into two distinct categories: 1) analysis of functional imprinting which examines the expression of a gene; and 2) analysis of epigenetic modifications of the DNA which must in some way influence expression of a gene. Although the germ-line methylation imprint is conserved during early pre- and postimplantation development it appears that methylation alone may not be enough to regulate the expression of imprinted genes [Latham et al., 1994]. Two earlier studies have examined the expression of imprinted genes in uniparental tissues [Mutter et al., 1993; Walsh et al., 1994]. Results of these studies demonstrated that both human and mouse uniparental tissues maintained expression patterns of *Igf2* and *Igf2r* which correlated with their parental origin. However, the expression of the H19 gene varied from the expected parental-origin-dependent pattern. Neither of these studies examined the methylation patterns of these genes in uniparental tissues, which may be independent of the level of expression detected. Recognition of a gametic imprint may require additional factors which are not expressed until after implantation, or may involve specific interactions between the maternal and paternal genomes.

The analysis of the *ZNF127* gene demonstrates a distinct pattern of hypo- and hypermethylated sites which depended on the parental origin of the alleles. The paternal pattern seen in sperm was conserved in the androgenetic CHM. This pattern was clearly different from normal somatic tissues, peripheral blood leukocytes, normal chorionic villi, and ovarian teratomas, which displayed a mix of developmentally modified methylation patterns. Since fetal development in CHM is arrested very early in gestation, the action of one or more maternal genes must be essential for further development. The absence of the maternal contribution results in the death of the embryo and the abnormal phenotype of CHM. The CHM may retain the paternal germ-line methylation imprint without further developmental modifications because they do not undergo normal embryogenesis.

We also observed a similar trend among most of the triploid conceptions; however, the germ-line methylation patterns were obscured by developmental modifications. The PHM displayed a level of DNA methylation which was consistent with the presence of an additional paternal allele and the NMT demonstrated a methylation pattern which was more consistent with an additional maternal allele [Driscoll et al., 1992]. Additional studies are necessary to confirm these findings since some of the DNA samples were obtained from cultured cells as opposed to fresh tissue. It has been shown that DNA methylation patterns can change during tissue culture (Driscoll, D.J., unpublished observation).

DNA methylation has already been shown to play a role in the regulation of expression of X-linked housekeeping genes. The CpG islands located 5′ to these genes are fully methylated on the inactive chromosome and completely unmethylated on the active chromosome [Driscoll and Migeon, 1990; Migeon et al., 1991]. Among the many potential CpG methylation sites within an imprinted gene, the specific sites which are imprinted probably become differentially methylated during gametogenesis. The unique methyl moieties must function to distinguish between the individual parental alleles. This has been observed in both the *Igf2r* gene [Stöger et al., 1993] and the imprinted transgene RSVIg*myc* (TG.A) [Chaillet et al., 1991]. The overall genomic methylation differences observed in the gametes appear to be erased during global demethylation which occurs during the preimplantation stage of development [Monk et al., 1987; Kafri et al., 1992]. The final adult methylation profile of somatic tissue is established by a second programmed demethylation event which occurs later in development [Migeon et al., 1991]. However, the specific methylation associated with the imprint might be maintained. Analysis of the transgene, RSVIg*myc*, suggests that the crucial stage in the development of an imprinted locus is the maintenance of specific parental methylation patterns during preimplantation development [Chaillet, 1994]. The imprint may be either 1) methylation, or 2) lack of methylation. Therefore, specific DNA-binding proteins which may be present in the early embryo may function to protect an imprinted site from developmental methylation changes. DNA methylation imprints have been found in all known functionally imprinted genes in the mouse [Surani, 1993; Razin and Cedar, 1994].

The mechanism by which the gametes can recognize and differentially methylate a specific imprinted site is not known. It is most likely directed by local cis-acting factors, such as a specific DNA sequence or chromatin confirmation. Based on the observations of other im-

printed genes it would appear that the specific signal does not need to be immediately adjacent to the coding sequence or the promoter, but may be in a distant flanking region or in an intron and may influence the conformation of the chromosome [Reis et al., 1994; Glenn et al., 1993a,b; Stöger et al., 1993; Brandeis et al., 1993; Sasaki et al., 1992].

Once the methylation imprint is established in the gamete and maintained in the embryo it may act as either a positive or negative regulator. DNA methylation has long been thought to inhibit transcription by altering the chromatin conformation and preventing the binding of transcription factors to the promoter. This is negative regulation of transcription in which the inactive allele is methylated. It also appears that methylation can play a role in the positive regulation of imprinted genes. In the *Igf2r* [Stöger et al., 1993], *SnrpN* [Glenn et al., 1993b], and probably *Xist* [Norris et al., 1994] genes it is the active allele which is modified. The DNA methylation modification in the *Igf2r* gene does not initially occur at the promoter, but rather at a CpG island 27 Kb from the start of transcription. The methylation of this CpG island in the body of the expressed paternal allele possibly inhibits a repressor protein from binding [Stöger et al., 1993].

In general, male germ cells are hypermethylated and the female germ cells are hypomethylated [Monk et al., 1987; Sanford et al., 1987; Driscoll and Migeon, 1990]. However, this does not appear to be the case for imprinted genes as indicated by both our results and the analysis of the *Igf2r* gene [Stöger et al., 1993]. Specific areas at the 5′ end of the *ZNF127* gene were unmethylated in sperm DNA and placental tissue from CHM versus the other tissues examined. Therefore, exceptions to the general level of methylation normally seen in sperm and oocytes may indicate a region which is imprinted and may be examined by detailed analysis of individual methylation sites within specific genes. In contrast to sperm, CHM, which are extraembryonic in origin, have low overall methylation [Manes and Menzel, 1981; Chapman et al., 1984; Sanford et al., 1985; Rossant et al., 1986], so the presence of discrete hypermethylated regions might imply loci where the imprint is maintained by targeted methylation. CHM thus provide a unique resource for studying this component of imprinting.

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